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14. ABSTRACT Androgen signaling via the androgen receptor, AR, is a key therapeutic target in prostate cancer. Our goal is to inhibit AR target genes that drive cancer but not normal cell growth to avoid resistance. Our hypothesis is that these genes differ in androgen response elements (AREs), with genes driving proliferation relying on consensus inverted repeats (cARE) and genes promoting differentiation relying on AR-selective direct repeats or half-sites (sAREs). To identify compounds that affect AR-DNA recognition, we performed a high-throughput screen for compounds eliciting differential AR activity on cARE vs. sARE reporters. Of 10,000 compounds, doxorubicin proved best at differentially affecting AR-driven gene expression, by interacting with DNA rather than directly with AR. This differential effect was pronounced at low doses of dox, leading to induction of sARE-driven genes in contrast to inhibition of cARE-driven genes, in multiple cell lines. Doxorubicin elicits DNA damage response, a pathway also influenced by AR. We used protein-DNA inter-action assays to show the differential effect of dox on AR binding <i>in vitro</i> , and extended this to show selectivity of AR binding <i>in vivo</i> by chromatin immunoprecipitation (ChIP) studies. Bioinformatic analysis of ChIP-seq data is ongoing to define genome-wide the set of genes sensitive to low dose dox.					
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Selective AR Modulators that Distinguish Proliferative from Differentiative Gene Promoters

1. INTRODUCTION: Prostate cancer (PCa) initially depends on androgens acting via the androgen receptor (AR). Potent new drugs block androgen synthesis and AR function, but resistance inevitably arises and AR regains control [1]. To reduce resistance, we have sought selective AR modulators (SARMs) that prevent expression of genes for cancer growth but allow expression of genes for differentiation. Some genes involved in differentiation rely on a selective Androgen Response Element (sARE) comprised of a half site or direct repeat element, whereas the consensus ARE (cARE) that drives most AR-responsive gene is an inverted repeat also recognized by other steroid receptors [2, 3]. We developed a high-throughput screen for compounds that elicit differential AR regulation based on distinct promoter elements. The strongest hit was doxorubicin (dox), one of the earliest chemotherapeutics, which inhibited cARE-driven reporters in preference to sARE-driven reporters. In prostate tumor cells, dox treatment inhibited cARE-driven endogenous AR target genes but, at low dose, selectively increased expression of sARE-driven AR targets. We showed by protein-DNA interaction studies *in vitro* that this selectivity relies on the greater disruption of AR binding to cAREs than to sAREs upon doxorubicin intercalation into DNA. Further, this effect was demonstrable in differential recruitment of AR to chromatin. The report below expands on these results, which have been repeated and validated for publication (*Specific Aim 2 of the proposal, subdivided into Tasks 2 and 3*). These results provide a foundation for a global view of distinct AR target gene sets and the extent to which they drive different cell growth behaviors (*to be explored in Task 4*). This may allow development of new prostate cancer therapies that modulate rather than completely block AR activity and thus delay resistance and produce fewer side effects.

2. KEYWORDS: androgen receptor, prostate cancer, antiandrogens, high-throughput screen, selective androgen receptor modulator, doxorubicin

3. ACCOMPLISHMENTS: The Statement of Work was revised 12/30/2016, with latter goals now focusing on doxorubicin as a validated drug screen hit eliciting differential AR action:

Task 1. Perform a larger screen for novel compounds using an improved protocol.

Task 2. Probe modulatory effect of doxorubicin on AR control and cell phenotype.

Task 3. Examine mechanism of selective effect of doxorubicin on AR action.

Task 4. Determine differential antitumor effects of low vs. high doses of doxorubicin in mice.

Differential effects of doxorubicin on endogenous AR target gene expression.

To explore selective modulation of AR action, we scanned a panel of androgen-responsive genes from published studies and our unpublished data, and selected several genes to characterize further for effects of doxorubicin in conjunction with the synthetic androgen R1881. Response to R1881 alone in LNCaP cells is shown in Table 1. Differential effects of dox were noted only on genes induced by R1881, not on those that are androgen-repressed (not shown). Several genes involved in DNA damage response or DNA repair did not respond to R1881 in LNCaP cells.

upregulated by R1881	ABCC4, CDK1, EAF2, FKBP5, HPGD, IGFBP3, KLF4, KLK1, KLK4, NCAPD3, NDRG1, NKX3.1, PSA, SARG, SGK1, TMEM16, TMPRSS2, VEGF, WIP1
downregulated by R1881	AQP3, AQP5
minimal response to R1881	GATA2, ID1, ID3, MAD2L1, MCM7, MME, Myc, NEDD4, P21, POLA2, POLE2, RAD9, RFC3

Table 1. Differential response of AR target genes to R1881. LNCaP cells were plated in 6-well plates with RPMI 1640 media with 10% FBS. 2 days later the cells were starved in media with 2% charcoal stripped serum for 24 hrs and then treated with 10 nM R1881 for 24 hrs. Total RNA was extracted and qRT-PCR was performed to quantify gene expression.

Many of the AR-induced genes were repressed by treatment with doxorubicin, as expected, but a subset of interest showed increased expression at low doses of dox. Gene response to R1881 plus dox is shown in Fig. 1 for genes induced by dox (in blue) compared to those repressed by dox (in yellow). Inhibition by dox of androgen induction occurred for classic cARE-driven targets, such as PSA, TMPRSS2, and FKBP5. Androgen-induced genes that were further induced rather than repressed by dox included SGK1 and SARG, known to be driven by sAREs [2, 4, 5], and some more recently identified “selective” AR responders (KLF4, IGFBP3). AREs accounting for the selectivity of the latter two genes have not yet been functionally validated.

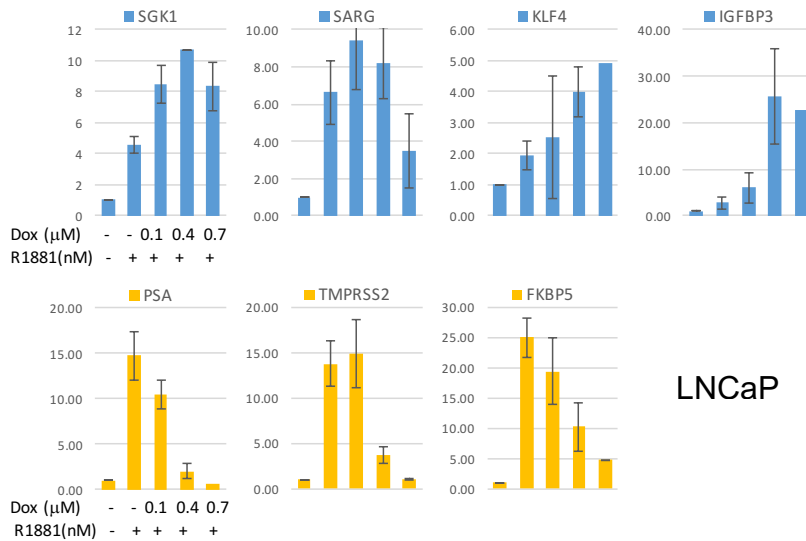


Fig. 1. Doxorubicin differentially regulates AR target gene expression in LNCaP cells. LNCaP cells were plated in 6-well plates with RPMI 1640 media + 10% FBS. 2 days later, the cells were starved in RPMI 1640 media with 2.5% charcoal-stripped serum for 24 hrs and then treated with 10 nM R1881 alone or plus dox for 24 hrs. Total RNA was extracted and qRT-PCR was performed to quantify gene expression. Dox stimulated gene expression of SGK1, SARG, KLF-4 and IGFBP3 (in blue, sARE-driven). Dox efficiently repressed expression of PSA, TMPRSS2, and FKBP5 (in yellow, cARE-driven). Results are from 3 independent experiments, with each point in triplicate.

To explore AR dependence of the differential dox response, we compared the effect in C4-2B cells (Fig. 2), a hormone refractory derivative of LNCaP with high AR levels that models castrate-resistant disease. Although C4-2B cells are androgen-insensitive when grown in mice, they retain some androgen response in tissue culture, although PSA response is atypical [6].

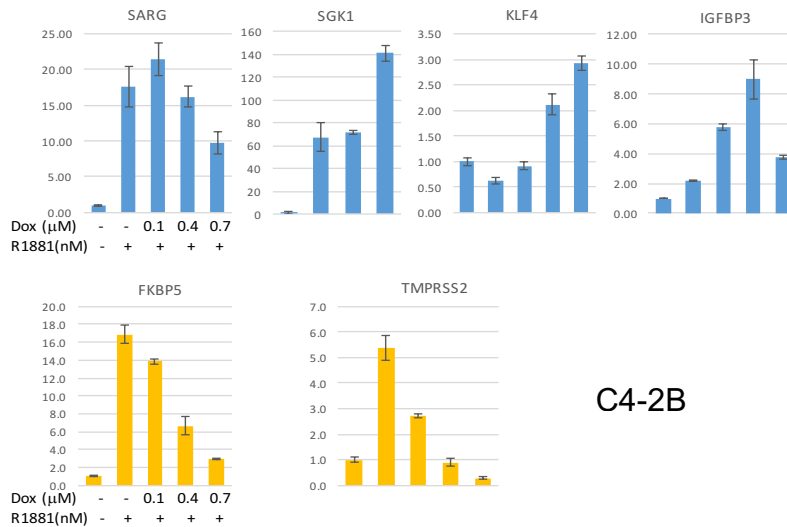


Fig. 2. Doxorubicin differentially regulates AR target gene expression in hormone-resistant C4-2B cells. C4-2B cells were plated in 6-well plates with RPMI 1640 media + 10% FBS. 2 days later, the cells were starved in RPMI 1640 media with 2.5% charcoal-stripped serum for 24 hrs and then treated with 10 nM R1881 alone or plus dox for 24 hrs. Total RNA was extracted and qRT-PCR was performed to quantify gene expression. Dox stimulated gene expression of SARG, SGK1, KLF-4 and IGFBP3 (sARE-driven genes in blue). Dox efficiently repressed expression of FKBP5 and TMPRSS2 (cARE-driven genes in yellow). Results are from 2 independent experiments, in triplicate.

The profile of gene response to R1881 plus doxorubicin was qualitatively similar between LNCaP and C4-2B cells. The hormone induction of the cARE-driven genes was less, as expected given the hormone-refractory nature of C4-2B cells (e.g., TMPRSS2 is induced 15x in LNCaP but only

5x in C4-2B cells). PSA regulation is complex and may be indirect in these cells [6] and for that reason is not shown. Interestingly, both SARG and SGK1, our hallmark validated sARE-driven genes, induce to higher levels with R1881 in C4-2B cells and increase with low-dose doxorubicin. This in part emphasizes distinct differences in AR regulation of sARE vs. cARE dependent genes.

Doxorubicin differentially affects AR recruitment to chromatin of AR target genes

To investigate the mechanism of the differential doxorubicin effect, we have been performing chromatin immunoprecipitation (ChIP) assays. Initial studies were done with the N-20 anti-AR antibody from Santa Cruz Biotechnology, which is no longer available. We compared several other antibodies, and found PG-21 from Millipore to be superior (Fig. 3).

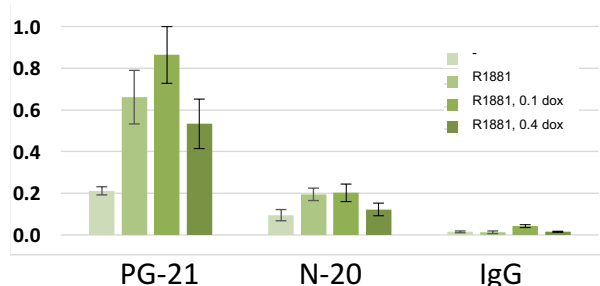


Fig. 3. Comparison of AR antibodies for ChIP assays.

LNCaP cells were plated, starved of hormone for 24 hrs, and then treated for 12 hrs with 10 nM R1881 and 0.1 or 0.4 μ M dox, as indicated in legend at upper right. Cells were crosslinked with formaldehyde and lysed to harvest chromatin, which was then sonicated to 300-1000 bp on ice. Immunoprecipitation was performed with 10 μ g PG-21 (Millipore), N-20 (Santa Cruz) or control IgG. DNA was purified and PCR was performed for the SARG promoter.

In addition to identifying a more efficient antibody, we optimized cell plating and induction conditions to improve the immunoprecipitation signal. Further, we used the HighCell# ChIP Kit from Diagenode; although expensive, this kit provides consistent high-quality data by using

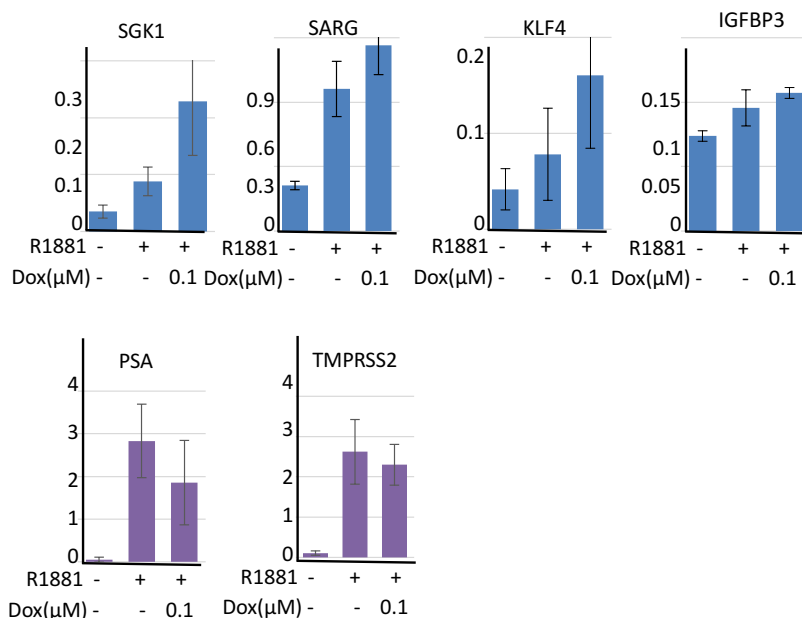


Fig. 4. Dox differentially affects AR recruitment to chromatin.

LNCaP cells were plated at 2.8×10^6 per 10 cm dish in complete media, and changed the next morning into starvation media (2.5% charcoal stripped serum). 12 hrs later R1881 (1 nM) and doxorubicin were added as indicated in the graph legends. After 12 hrs of induction, cells were collected and crosslinked with formaldehyde, using 3 plates per sample. The HighCell# Chip kit and protocol were followed, using a colleague's Bioruptor for more controlled shearing. 10 μ g antibody was used per sample. Recovered DNA was subjected to qRT-PCR with primers targeting AREs in the promoter/enhancer regions of specific genes as indicated. Results shown are combined from 2 independent experiments.

magnetic beads to improve handling, reproducibility and recovery of immunoprecipitated DNA. As shown in Fig. 4, this allowed us to compare a broad array of genes driven by cAREs or sAREs with respect to AR recruitment to chromatin. sARE-driven genes (SGK1, SARG) and the AR-specific genes (KLF4, IGFBP3) showed enhanced AR recruitment by 0.1 μ M dox over the R1881-induced level, whereas cARE-driven genes (PSA, TMPRSS2) showed less AR recruitment. This mirrors the effects seen at the transcriptional level (see Fig. 1). For some genes that were only

modestly upregulated by low-dose dox, such as NDRG1, there was no significant difference upon treatment detected by ChIP, suggesting the AR regulation may be indirect (not shown).

Results thus far of the ChIP experiments showed strong accord between differential effects of dox on recruitment of AR to chromatin and the resulting transcriptional effects. That is, low dose dox induced a small set of androgen-responsive genes while most AR targets were repressed by dox at all doses. For some of the dox-induced genes selective AR response elements are known and the others appear to be AR-specific in response with sAREs yet to be identified. General effects of dox on genomic activity have been noted, largely with regard to active vs. inactive genes [7, 8], but gene-specific or response element-specific effects have not been studied. Since dox is still commonly used in chemotherapy, including in prostate cancer, this could prove informative for heterogeneity in drug response. An intriguing possible link between the dox effect noted here and AR is via the DNA damage response, which is induced by dox treatment and subsequently induces AR, promoting expression of a program regulating DNA repair [9]. Thus identification of the set of differentially affected AR-responsive genes would be important and address several aspects: the mechanism (is this effect mediated by targeting distinct “selective” AREs?); the resultant biology (do these genes direct different growth behavior in tumors, and by what pathways?); and what might be long-term consequences of low dose dox treatment (is low dose dox an alternative to consider for particular stages of prostate cancer?). Therefore we began to scale up for ChIP-seq studies as a means to identify genome-wide targets of AR plus dox action.

ChIP-seq studies require larger amounts of sample material than conventional single-gene ChIP assays, so LNCaP cells were expanded and plated in 15 cm rather than 10 cm dishes. Surprisingly, this change led to a loss of the differential effect of dox on gene regulation (Fig. 5). While SARG and SGK1 showed enhanced AR recruitment to chromatin with 0.1 μ M dox for cells grown in 10 cm dishes, there was no enhancement by low dose dox in 15 cm dishes (Fig. 5A). This insensitivity to dox also was evident at the transcriptional level as shown by qRT-PCR of cellular RNA from cells grown in 6-well plates, 10 cm or 15 cm dishes (Fig. 5B). Additional

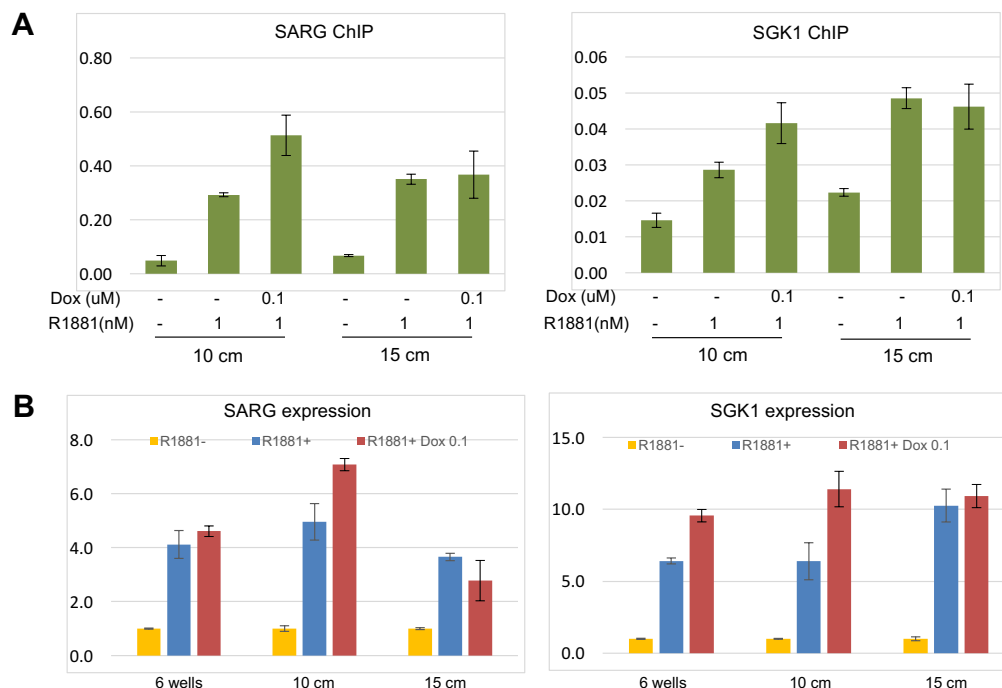


Fig. 5. Effect of cell growth conditions on sensitivity to low dose dox treatment. LNCaP cells were seeded and treated as previously (see above), with samples harvested for chromatin immunoprecipitation (A) or RNA assay (B). Shown are results for SARG and SGK1. While AR recruitment to chromatin and gene induction are evident and as expected in 10 cm and 6-well plates, the enhancing effect of low dose doxorubicin is not seen in 15 cm plates.

studies suggest this was due to greater cell density in 15 cm dishes by treatment end, and therefore a likely influence of hypoxia (data not shown). Since hypoxia can induce the DNA damage response [10], the combined effect of hypoxia and low dose dox is likely greater than low dose dox alone, tipping the balance to inhibition rather than increased AR effect for genes showing selective response to AR (sARE-driven). We thus invested significant effort attempting to adjust the cell plating density and optimize treatment conditions in order to be able to treat and harvest sizable cell numbers for ChIP-seq with reproducible results. We ultimately found results to be most consistent and reproducible with 10 cm rather than 15 cm dishes, and so proceeded with using a larger number of plates despite less convenience in handling.

For ChIP-seq, robust controls are critical to assess transcription factor binding, given the thousands of sites that may be occupied dependent on factor, only some of which may be functionally significant. We chose TBP as an informative control for AR binding since it may more closely indicate active genes than PolII, which binds inactive enhancers and paused promoters as well as actively transcribing genes [11]. Several antibodies are available for TBP that give robust signals like for PolII but are more reflective of actual transcription. Cells were plated in triplicate 10 cm plates, for each of 3 antibodies (AR, TBP, IgG), and for 7 treatment groups that cover effects of hormone alone, dox alone, and hormone plus several dox concentrations (number of samples were limited by cost of bar-coded DNA library preparation, deep sequencing and bioinformatic analysis). Results for single gene ChIP are shown in Fig. 6 (next page). As in previous experiments, the sARE-driven gene SGK1 shows increased recruitment of AR to its promoter with low-dose dox; SARG shows similar AR recruitment with low dose that slowly declines as the concentration is increased. In contrast, the cARE-driven genes TMPRSS2 and PSA show significant reduction in AR recruitment at the lowest dose of dox tested and further reduction as the dose is increased. Effects of dox alone are marginal. Importantly, TBP gives a robust signal, increasing greatly as expected with hormone treatment that stimulates transcription, but dropping dramatically with dox treatment, for all genes. Thus the selectivity in recruitment for sARE vs. cARE genes is not evident, and dox-selective effects vary depending on the transcription factor. This emphasizes that the effect of doxorubicin on these promoters is specific to AR and is not just a general effect, for example on chromatin rearrangement. This may suggest further that the effect on increased transcription at low dose for some genes is locally initiated and transmitted by proteins interacting with AR, rather than transduced along the chromosome.

Treatment Groups for ChIP-seq

	<u>Hormone</u>	<u>Doxorubicin</u>
1.	-	-
2.	-	0.1 μ M
3.	-	0.7 "
4.	1 nM R1881	-
5.	"	0.1 μ M
6.	"	0.4 "
7.	"	0.7 "

DNA from these chromatin preparations was converted to libraries for ChIP-seq, using the Maestro KAPA DNA Library Preparation Kit from Illumina. In brief, indexed adapters were ligated onto sheared DNA fragments in several steps using the Sciclone NGS Workstation, to produce libraries appropriate for multiplexing and paired-end sequencing on the Illumina platform. Libraries were checked for quality control features (size distribution and concentration of fragments in the appropriate size range), using LabChipGX analysis. Libraries for all 21 samples were made, and those immunoprecipitated with AR and TBP antibodies were sent for DNA sequencing, with the IgG control held in reserve should problems arise. The sequencing has been completed, with read depths of at least 30M per sample, and all have passed QC. We are now awaiting analysis from the bioinformatics team. Although the wait periods are long for sequencing and bioinformatics analysis, the wealth of data expected is well worth it.

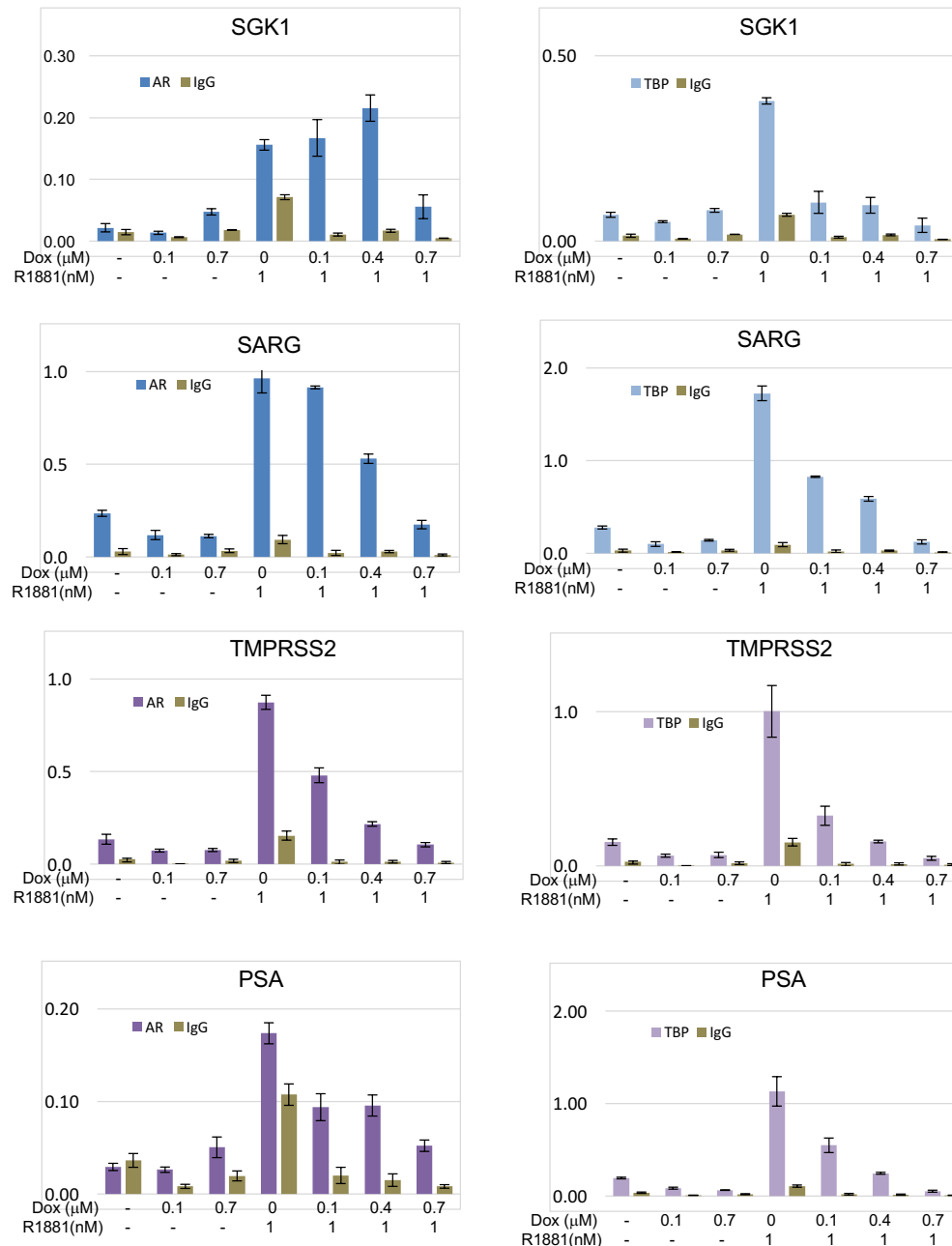


Fig. 6. Gene-selective and dose-dependent effect of dox on AR recruitment to target genes. LNCaP cells were seeded at 2.8×10^5 cells per 10 cm dish, with 3 dishes per treatment point, in complete RPMI-1640 medium free of phenol red. In the morning, media was changed to starving media (2.5% CSNS). 12 hrs later, R1881 and/or doxorubicin were added in accord with treatment group. 12 hrs later, cells were collected for ChIP following the protocol for the HighCell#ChIP kit. For immunoprecipitations, 10 μ g antibodies were used per point: PG-21 (Millipore), TBP (Abcam) and IgG (Santa Cruz Biotechnology). Shown here are results from DNA prepared from an aliquot of each immunoprecipitate assayed by conventional ChIP for two sARE-driven genes (SGK1, SARG) and two cARE-driven genes (TMPRSS2, PSA) for AR and TBP binding in response to no treatment, dox alone, R1881 alone, or both, as indicated for each graph.

Differential effect of doxorubicin on cell proliferation and AR target gene expression is sustained with long-term treatment

For clinical relevance and benefit of low dose dox, such effect must have a longer duration than the day or so of most tissue culture experiments. Prior to a preclinical test of the ability of dox to repress androgen-dependent xenograft tumor growth, or to redirect growth phenotype, we examined the effect of longer dox treatment on cell proliferation and gene expression, for both LNCaP and LAPC4 cells. LAPC4 cells were compared as a preferable line to use for xenograft experiments since they establish tumors more efficiently than LNCaP and are capable of metastasis. Moreover, they represent an earlier tumor stage than LNCaP cells and therefore are more comparable to patients likely to benefit from low dose dox treatment.

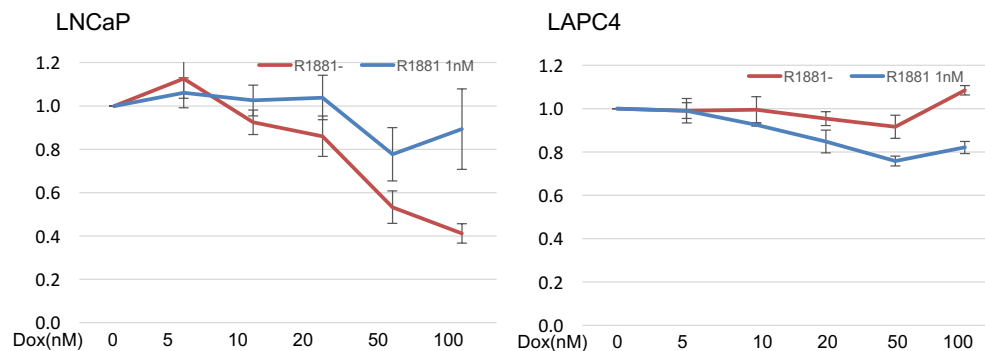


Fig. 7. Effects of low dose dox on LNCaP and LAPC4 cell proliferation. 5×10^3 cells were seeded per well in 96-well plates, 5 wells per condition, in complete media. 2 days later media was changed to 2.5% CSNS, and the next day, compounds were added as indicated (orange line, no R1881; blue line, 1 nM R1881; dox concentration along bottom). MTT assays were performed after 7 days.

LNCaP and LAPC4 cells were treated with dox, in the presence or absence of 1 nM R1881, focusing on lower dox doses where differential effects on gene expression and AR recruitment were observed (Fig. 7). Effects on proliferation were not detected at 1-3 days (not shown). By 7 days of treatment, 100 nM (0.1 μ M) dox inhibited proliferation in LNCaP cells in the absence of R1881, likely reflecting the dependence of these cells on androgen for growth; the dox effect in the presence of R1881 was less consistent. In LAPC4 cells, dox was modestly more inhibitory in the presence than absence of androgen. Thus low levels of dox are not significantly toxic to cell growth. We next evaluated effects on gene expression with 7 days of treatment for sARE-driven genes (SGK1, SARG, KLF4, IGFBP3) and cARE-driven genes (PSA, TMPRSS2) (Fig. 8).

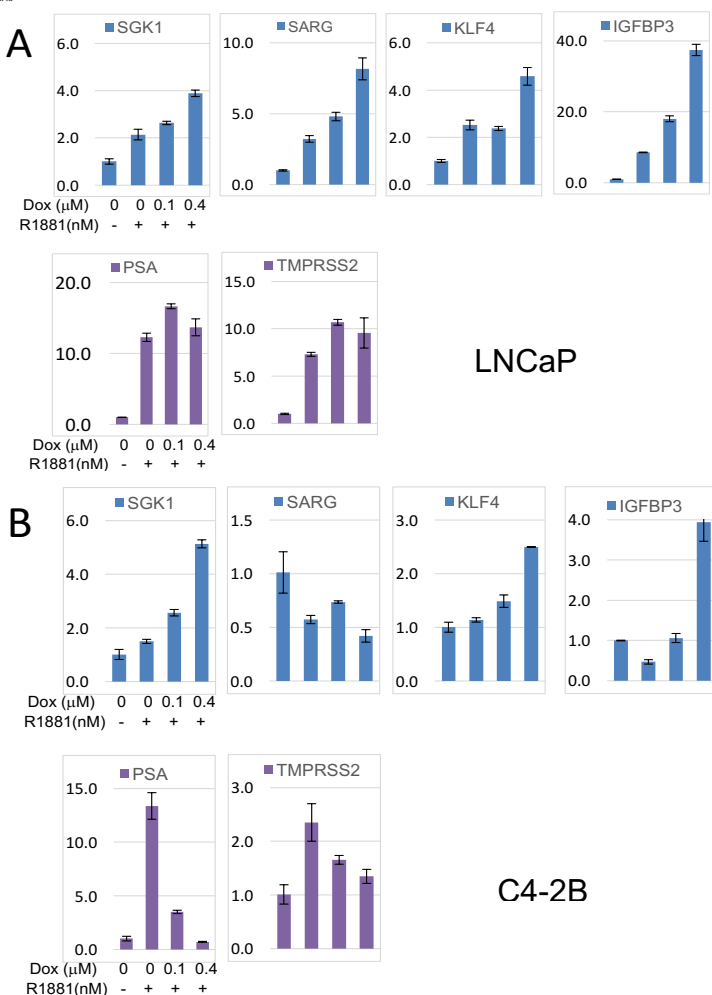


Fig. 8. Differential effects of low dose dox on AR target gene expression are sustained during 7 days of treatment. LNCaP cells (A) or LAPC4 cells (B) were plated into 6-well plates in full media. After 2 days, media was changed to phenol-red free media with 2.5% charcoal stripped serum for 24 hrs, and then compounds were added. In each panel of 4 treatments, the first sample received vehicle alone, the next 3 received 1 nM R1881, the 3rd sample also received 1 μ M dox, the 4th sample 0.4 μ M dox (as in legend below left-most panels). Fresh media and compounds were changed on the 4th day. After 7 days, RNA was extracted and qRT-PCR was performed as before to quantify gene expression.

With longer term treatment, the enhancement of AR induction by dox at low doses is still evident in LNCaP cells for sARE-driven genes (upper panels) after 7 days, in a pattern similar to that seen after 1 day of stimulation (compare Fig. 1). An inhibitory effect on cARE-driven genes, PSA and TMPRSS2, is less evident, perhaps in part because these experiments used 1 rather than 10 nM R1881, in accord with greater physiological relevance. Interestingly in C4-2B cells, the androgen induction of sARE-driven genes and TMPRSS2 is much less, but there is still a clear enhancement by low-dose dox for SGK1 and increased expression for KLF4 and IGFBP3 at 0.4 μ M dox. As predicted, dox is inhibitory to cARE-driven PSA and TMPRSS2 in C4-2B cells. Thus differential regulatory effects remain in evidence after a week of treatment, although with some differences compared to short term effects. This could be due to downstream or secondary effects. These differences will be analyzed in greater detail in xenograft experiments but overall justify pursuit of long-term benefits in vivo.

Summary. Differential effects of doxorubicin action on AR-responsive genes have been characterized in greater depth for a broader array of genes in multiple prostate cancer cell lines. A small subset of AR-responsive genes, with SGK-1 and SARG serving as hallmarks, model enhanced expression over AR induction caused by low doses of dox, due to selective AR response elements. This is in contrast to most AR target genes driven by consensus AR binding sites that are very sensitive to dox and show inhibition at all concentrations. This differential dox effect occurs in a variety of androgen-sensitive prostate cancer cell lines, with some cell-specific differences. The effect is evident on AR recruitment to chromatin, supporting the notion that the primary and direct effect of dox on AR binding is at the level of transcription. Further, this effect is sensitive to other cell regulatory pathways, as demonstrated by the abrogation of dox enhancement under hypoxic conditions. Large-scale chromatin immunoprecipitations were performed, libraries prepared and deep sequencing has been performed; we are awaiting bioinformatic data to proceed with the ChIP-seq analysis. The effect of low dose dox is sustained over many days of treatment, and will now be tested in xenograft experiments. Experiments have been repeated and expanded such that the addition of ChIP-seq and xenograft data will allow us to complete and publish this project.

KEY RESEARCH ACCOMPLISHMENTS

- Expression of additional genes, in several cell lines, have been tested to confirm that cARE-driven genes are efficiently inhibited by dox whereas sARE-driven genes are upregulated, particularly at low dose, with some genes showing intermediate response.
- AR recruitment to chromatin parallels the differential effect noted at the RNA level; this effect is AR-specific since TBP shows inhibition by dox for all promoters, including those upregulated by low dose dox. ChIP-seq has been performed, with analysis in progress.
- The promoter-specific differential effect of dox is not transient but is sustained over a week of cell culture, providing a basis for preclinical testing in xenograft tumor growth.

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Opportunities for Training and Professional Development

Dr. Shihan He began on this project as a postdoctoral fellow and was appointed as a Research Specialist in 2016. Since the project had an original end date of July 29, 2017, and there was an unexpected delay in processing the no-cost extension, he sought other positions and left on short notice for a start-up data analysis company in North Carolina, on June 9, 2017. While it would have been preferable for him to stay to see the project through to its conclusion, it is gratifying that he received several offers and I wish him the best in his new position.

Dissemination of Results

Nothing to report during this period.

Next Reporting Period

Analysis of the ChIP-seq data will be accomplished with the imminent delivery of bioinformatics analysis from the core, complementing any experiments of Tasks 2 and 3 remaining to be completed. Mouse xenograft experiments will be performed, tissue and RNA analyzed, to complete Task 4. Once all the data is together, we expect this to be a novel and important study.

4. IMPACT

Nothing was reported during this period. Once ChIP-seq and xenograft data are analyzed we will write up results for publication and submit abstracts to meetings for presentation.

5. CHANGES/PROBLEMS

Since revising the SOW for the last annual report, we have been able to focus more on attainable goals. Nevertheless, progress was slow, in part due to unexpected problems in optimizing conditions for large-scale ChIP experiments. This was time consuming particularly for LNCaP cells that grow slowly and adhere poorly to the dish. In addition, Dr. He spent significant time working on job searches and left the lab before the end of the reporting period.

6. PRODUCTS

Nothing to report during this period.

7. PARTICIPANTS

Name: Diane M. Robins, Ph.D.
Project Role: P.I.
Research ID (ORCID): 0000-0001-6727-6309
Person Mo.: 1
Contribution: Dr. Robins conceives and oversees the experiments and reports the results
Funding: 10% from this award, 2.5% from the SPORC

Name: Michele Brogley
Project Role: Res. Assistant
Research ID: n/a
Person Mo.: 5
Contribution: Ms. Brogley assists with cell culture, molecular assays and lab managing
Funding: Ms. Brogley's effort on this project is at 40%

Name: Shihan He, Ph.D.
Project Role: Research Associate
Research ID (ORCID): 0000-0001-5806-8583
Person Mo.: 12, until 06/09/17 when he moved to a new position
Contribution: Dr. He performed cell gene expression, cell growth and ChIP assays
Funding: Dr. He was funded by this DOD grant until 06/09/17

8. SPECIAL REPORTING REQUIREMENTS – N/A

9. APPENDICES – N/A